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Thank-You!

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Hemodynamic forces are complex regulators of endothelial gene expression

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ABSTRACT **Vascular endothelial cells, by virtue of their unique anatomical position, are constantly exposed to the fluid mechanical forces generated by flowing blood. In vitro studies with model flow systems have demonstrated that wall shear stresses can modulate various aspects of endothelial structure and function. Certain of these effects appear to result from the regulation of expression of endothelial genes at the transcriptional level. Recent molecular biological studies have defined a "shear stress response element" (SSRE) in the promoter of the human platelet-derived growth factor (PDGF)-B chain gene that interacts with DNA binding proteins in the nuclei of shear– stressed endothelial cells to up–regulate transcriptional activity. Insertion of this element into reporter genes also renders them shear-inducible. Further characterization of this and other positive (and negative) shear-responsive genetic regulatory elements, as well as their transactivating factors, should enhance our understanding of vascular endothelium as an integrator of humoral and biomechanical stimuli in health and disease.—Resnick, N., Gimbrone, M. A., Jr., Hemodynamic forces are complex regulators of endothelial gene expression. *FASEB J.*, 9, 874–882 (1995)**

Key Words: endothelium • shear stress • gene expression

THE PULSATILE FLOW OF BLOOD through the branched tubular network of the mammalian circulatory system generates biomechanical forces that act on blood vessels to modulate their intrinsic structure and function (1). These hemodynamic forces, which include hydrostatic pressures, cyclic strains, and frictional wall shear stresses, constitute a special category of biophysical stimuli that, in addition to better characterized biochemical stimuli, can elicit important biologic responses in the cells that compose the blood vessel wall. Because of the central role played by the endothelial cell in the economy of the vessel wall (2), hemodynamically induced responses in this cell type in particular can have important physiologic as well as pathophysiologic implications. How vascular endothelial cells sense and transduce changes in their biomechanical environment into biological responses has become a subject of intense investigation (3). In this brief review, we focus on recent insights into the molecular mechanisms that link hemodynamic forces, in particular wall shear stresses, to the regulation of endothelial gene expression. We also consider some of the unanswered questions raised by the complexities of this biological process.

ENDOTHELIUM-DEPENDENT VASCULAR ADAPTATIONS TO BLOOD FLOW

In vivo, large blood vessels, such as elastic and muscular arteries, can undergo dramatic adaptations in response to both acute and chronic alterations in blood flow that appear to be endothelium-dependent (4). Certain of these changes, such as vasodilation and vasoconstriction, reflect alterations in the rates of production of endothelial-derived mediators, such as prostacyclin and nitric oxide, which act locally to modulate the tone of vascular smooth muscle (5–8). Typically, this level of regulation involves changes in the availability of substrate or the activity of rate-limiting enzymes, and appears to serve a homeostatic function in maintaining constancy of tissue perfusion in response to fluctuating metabolic demands. More chronic changes in blood flow, such as those resulting from disease-related or experimentally induced vascular narrowings and/or shunts, can elicit more profound structural remodeling involving cell proliferation and cell death (e.g., apoptosis), as well as hypertrophy of extracellular matrix. These longer–term structural modifications, which presumably reflect a complex balance of locally generated growth inhibitory and growth stimulatory substances, also appear to be endothelium-dependent (9).

The influence of local hemodynamic factors extends down to the level of the individual endothelial cells that compose the continuous vascular lining. In simple tubular portions of the arterial vascular tree, endothelial cells exhibit an ellipsoidal shape and are coaxially aligned with the direction of blood flow, whereas near branch points the

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[2]Abbreviations: PDGF-B, platelet-derived growth factor-B chain; SSRE, shear stress– responsive element; BAEC, bovine aortic endothelial cells; HUVEC, human umbilical vein endothelial cells; TFGβ, tranforming growth factor–β; ICAM–1, intercellular adhesion molecule–1; MCP–1, monocyte chemotactic protein–1; ecNOS, endothelial cell nitric oxide synthase; IERGs, immediate–early response genes.

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shape of individual cells is more polygonal and without any obvious orientation, consistent with the local temporal and spatial fluctuations in flow . This classically described morphologic pattern has led to the suggestion that the endothelial cell might function as a sensor of local fluid mechanical forces (10).

Given the recent appreciation of the pivotal role of the endothelial cell in the pathophysiology of various disease processes, including thrombosis, acute and chronic inflammation, hypertension, and atherosclerosis (2), the participation of endothelial-mediated hemodynamic influences in the pathogenesis of vascular disease is also receiving increased attention. The nonrandom distribution of early atherosclerotic lesions (11) observed both in the natural disease process in humans and in experimental animal models has long been cited as suggestive evidence, although the exact nature of the pathogenetic fluid mechanical factors (high vs. low wall shear stresses, flow disturbances, stagnant boundary layer) (12, 13) remains an area of ongoing debate.

IN VITRO FLUID MECHANICAL MODEL SYSTEMS

To explore the hypothesis that fluid mechanical forces could act as direct stimuli modulating vascular endothelial structure and function, several laboratories undertook in the early 1980s the development of in vitro model systems to simulate the in vivo biomechanical environment of the vascular endothelial cell (14–19). This experimental strategy has permitted deliberate control of selected mechanical parameters (e.g., amplitude, duration, and spectral properties of the applied force), as well as more precise measurement of biological responses at the cellular level.

For flow simulation studies, two basic systems have been designed. The first is a relatively simple parallel-plate flow chamber (**Fig. 1***A*), in which well–developed laminar flows are generated by a pump device over a confluent endothelial monolayer grown on a transparent coverslip. In this system, wall shear stress, the tractive force imparted by the movement of a viscous fluid over the luminal endothelial surface, is a linear function of the volume flow rate through the channel. Using a closed sterile system with periodic replenishment of medium, cells can be maintained under defined flow for several days. Coupled with a phase-contrast microscope or microfluorimeter, this system permits visualization of shear stress–induced changes in cell morphology or live-time functional analyses (e.g., ionized cytosolic calcium measurements with fluorescent reporters) (20–22). The second system is a modified cone-plate viscometer (14) in which shear stresses are produced in a layer of fluid contained between a stationary base plate and a rotating cone (**Fig. 1***B*). By adjusting cone angle, medium viscosity, and cone rotation speed, a broad dynamic range of shear forces (typically in the range of 1 to 50 dynes/cm^2) can be generated, in both laminar and turbulent flow patterns (23). Confluent endothelial monolayers grown on small coverslips and mounted at different positions in the base plate allow multiple samplings of the flow field. Alternatively, a single, large plastic plate insert can be substituted, thus enabling scale-up of sample size for molecular biological analyses. Unsteady (oscillatory) flows can be generated by tipping the cone with respect to the axis of rotation. In addition, small, defined areas of disturbed flow can be created on each coverslip by the addition of a barrier to the primary flow in the form of a step (Fig. 1*B*, inset). The latter system generates a separated flow region downstream with complex secondary flows and large spatial variations in the shear gradient (24), thus mimicking the disturbed laminar flow patterns that occur in vivo near arterial bifurcations (11) (Fig. 1*C*).

To simulate the rhythmic deformation of the arterial wall associated with systolic-diastolic pressure changes, endothelial cells have also been grown on distensible membranes and subjected to rapid (60 cycles/min) stretching in specially designed cyclic strain apparatuses (25). More recently, hydrostatic pressures also have been applied to endothelial cultures in the absence of fluid flow (26).

Of these various hemodynamic forces, perhaps the most extensively investigated to date has been the influence of fluid shear stresses applied to confluent monolayers of cultured large vessel endothelial cells. An important biological validation of this in vitro model system is the morphologic observation that unidirectional laminar shear stresses applied to cultured endothelial monolayers induce a time- and force-dependent cell shape change and alignment that is reversible upon cessation of flow (15, 19, 27, 28). This shear-induced shape change is accompanied by reorganization of the cytoskeleton (29), thus mimicking the morphology of aortic endothelium in vivo (30).

ENDOTHELIAL RESPONSES TO DEFINED FLOW STIMULI IN VITRO

In addition to morphologically evident changes, cultured endothelial cells exhibit a diverse array of biochemical responses to defined flow stimuli (see refs 3, 31 for reviews). These range from virtually instantaneous electrochemical responses (e.g., membrane hyperpolarization secondary to ion channel activation) to more delayed changes in gene expression. Basic cellular processes, such as the rate of formation of pinocytotic vesicles (16) or cell proliferation (32), as well as the secretion of specialized effector molecules such as fibrinolytic activators and growth factors (33–36), show complex patterns of response to experimental flow conditions designed to mimic the force amplitude as well as spectral properties of in vivo wall shear stresses. For example, application of unidirectional laminar shear stresses to confluent endothelial monolayers tends to suppress cell replication (37), whereas turbulent shear stresses of comparable time-averaged magnitude trigger the majority of cells to enter cell cycle and divide (32). Cultured endothelial cells in a disturbed laminar flow field exhibit a complex spatial pattern of response, with maximal perturbation in the flow reattachment region where the most ex-

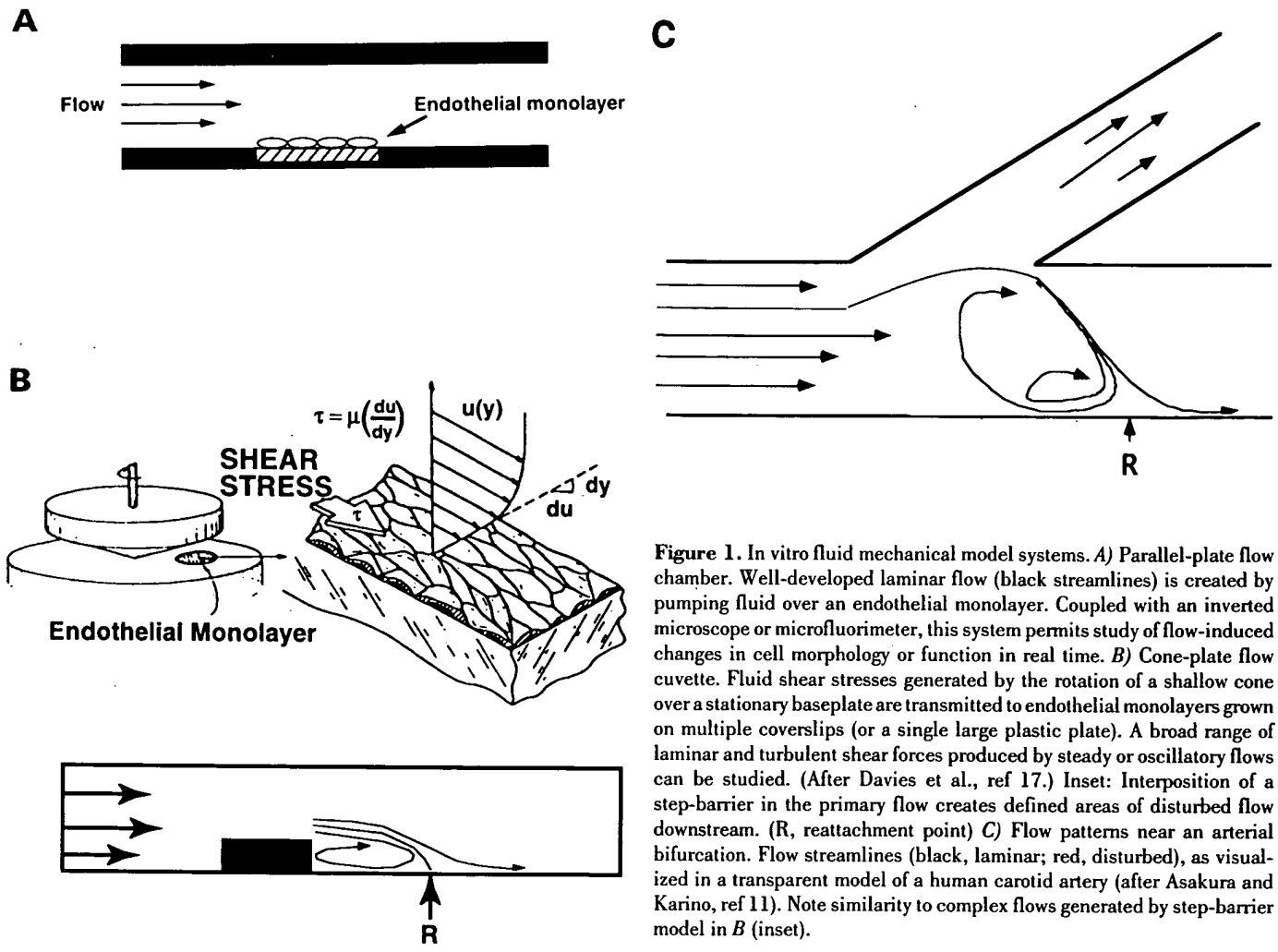


Figure 1. In vitro fluid mechanical model systems. *A)* Parallel-plate flow chamber. Well-developed laminar flow (black streamlines) is created by pumping fluid over an endothelial monolayer. Coupled with an inverted microscope or microfluorimeter, this system permits study of flow-induced changes in cell morphology or function in real time. *B)* Cone-plate flow cuvette. Fluid shear stresses generated by the rotation of a shallow cone over a stationary baseplate are transmitted to endothelial monolayers grown on multiple coverslips (or a single large plastic plate). A broad range of laminar and turbulent shear forces produced by steady or oscillatory flows can be studied. (After Davies et al., ref 17.) Inset: Interposition of a step-barrier in the primary flow creates defined areas of disturbed flow downstream. (R, reattachment point) *C)* Flow patterns near an arterial bifurcation. Flow streamlines (black, laminar; red, disturbed), as visualized in a transparent model of a human carotid artery (after Asakura and Karino, ref 11). Note similarity to complex flows generated by step-barrier model in *B* (inset).

treme fluctations in wall shear rate occur (24). This and other observations (16) suggest that the endothelial cell may be especially responsive to change in its fluid mechanical environment.

Many flow-induced responses resemble classic receptor-mediated, second-message coupled changes in the activity of intracellular enzymes or extracellular effector molecules. Indeed, studies of adenosine nucleotide–induced endothelial responses have pointed to a role for flow–related alterations in the mass transport of agonists in the vicinity of cell–surface receptors (20, 21). Nonetheless, in addition to these indirect effects of fluid movement on boundary layer transport phenomena, there appear to be numerous examples of biological responses mediated by direct mechanical stimulation of endothelial cells (31, 38). When surveying these force-mediated endothelial responses, one is initially struck by their diversity, not only in terms of their functional implications but also their temporal pattern. As outlined in **Table 1** (and considered in greater detail in refs 3, 31), endothelial responses to fluid shear stresses can be grouped broadly into three temporal categories: immediate (seconds to minutes), intermediate (minutes to hours), and delayed (hours to days). The earliest detectable events, such as ion movement and activation of polyphosphoinositide metabolism, presumably are very proximal manifestations of the biomechanical transduction process and appear causally linked to other "immediate" metabolic effects, such as changes in nitric oxide production that might occur in vivo with acute alterations in blood flow. At the other end of the temporal spectrum, delayed responses in cultured endothelial monolayers such as cell shape change, cy-

TABLE 1. *Flow-mediated endothelial responses*

Immediate (seconds-minutes)	Intermediate (minutes-hours)	Delayed (hours-days)
Biomechanical Transduction events	Primary biological Responses	Cellular Adaptations
- ion channel activation	- endocytosis	- cell shape change & alignment
- cytosolic calcium changes	- cell replication	- reorganization of cytoskeleton & focal attachments
- second messenger cascades	- gene regulation	- extracellular matrix remodeling

toskeletal reorganization, and extracellular matrix remodeling represent more chronic cellular adaptations, analogous to certain chronic structural changes that blood vessels undergo in response to altered hemodynamics in vivo. The intermediate category (onset measured in minutes to hours) consists of primary biological responses that are detectable at the cellular or molecular level. These include cell membrane deformation (reflected in transient changes in pinocytotic vesicle formation), changes in cell cycle kinetics, and also the up- and down-regulation of a vast array of gene products. Many of the latter represent key components of pathophysiological effector systems located in the vascular endothelium that modulate hemostasis and thrombosis, vascular tone, cell growth, and inflammatory and immune reactions. Although attention initially was focused on the functional implications of individual effector molecules, these flow-regulated endothelial products have proved to be useful experimental paradigms for investigation of the regulation of endothelial gene expression by biomechanical forces.

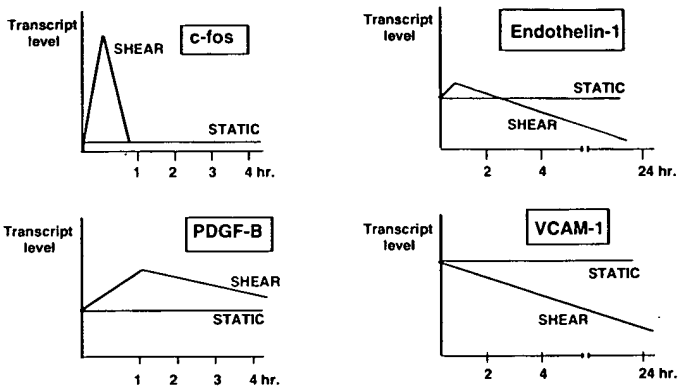


Figure 2. Patterns of endothelial gene regulation by laminar shear stress. Four typical patterns of transcriptional regulation of endothelial genes induced by an in vitro continuous laminar shear stress stimulus are depicted, using c-fos, PDGF-B, endothelin-1, and VCAM-1 as examples. Steady–state mRNA levels are compared, as a function of time of exposure to the shear stimulus (SHEAR), to the basal expression of each gene under no flow (STATIC) conditions.

PATTERNS OF ENDOTHELIAL GENE REGULATION BY FLUID SHEAR STRESSES

In 1990 Diamond and co–workers (33) demonstrated that steady–state transcript levels for a fibrinolytic enzyme were up–regulated in cultured human umbilical vein endothelial cells after their prolonged (24 h) exposure to an elevated level (25 dynes/cm^2) of laminar shear stress. This increase in mRNA correlated with enhanced production of the secreted protein product, tissue plasminogen activator, thus documenting for the first time that a defined fluid mechanical force could influence endothelial gene expression. Subsequently, other endothelial genes also have been shown to be regulated by fluid shear stresses (as well as other biomechanical forces), but the observed force dependencies and kinetic profiles of their various patterns of response suggest that the molecular mechanisms linking the externally applied force to genetic regulatory events in the nucleus are complex. Some examples of typical response patterns of endothelial genes to continuous fluid shear stress are schematically represented in **Fig. 2.** Steady–state mRNA levels for four representative genes are plotted as a function of time after the application of a continuous laminar shear stress stimulus. The first example is c-fos, a proto-oncogene that encodes a DNA binding protein of the AP-1 family (39), which can function in the transactivation of other genes. Under static (no–flow) conditions transcript levels are low, and in response to shear there is a rapid induction to a high level (20–fold basal), followed by an equally rapid return to baseline (40, 42). This rapid up-down pattern is typical of the response of these immediate–early response genes (IERGs).[2] The second example is platelet-derived growth factor-B chain (PDGF-B), a secreted growth factor and vasoconstrictor protein that has been implicated in wound healing and atherogenesis. In cultured endothelial cells, significant transcript levels are present under static conditions and therefore induction by shear stress is less pronounced (four- to fivefold). Onset is relatively rapid (less than 1 h) and transcript levels remain elevated for several hours before returning to baseline (34–38). Several other shear-inducible endothelial genes also follow this pattern (e.g., endothelial cell nitric oxide synthase [ecNOS], c-jun, PDGF-A, monocyte chemotactic protein-1 (MCP-1), transforming growth factor-beta [TFG-β], basic fibroblast growth factor, tissue plasminogen activator, intercellular adhesion molecule-1 [ICAM-1]) (33–36, 42–45). Certain of these genes (e.g., PDGF-A, ecNOS), however, exhibit lower basal transcript levels under static conditions than PDGF-B, and thus manifest a more substantial shear induction. In at least one case—MCP-1—an initial positive induction phase is followed by a more sustained depression below static levels (45). A third response pattern is illustrated by endothelin-1, a secreted vasoconstrictor, and also by thrombomodulin, an endothelial surface protein involved in hemostasis (46–47). Both show basal expression under static culture conditions and undergo an initial, modest shear induction (1.5–fold, 30 min) but then are significantly down-regulated, returning to baseline after only 24–48 h. A fourth response pattern is exhibited by VCAM-1, an inducible endothelial-leukocyte adhesion molecule that has been implicated in inflammation and atherogenesis. In static HUVEC cultures, which typically exhibit little VCAM-1 mRNA in the absence of endotoxin or cytokine stimulation, shear stress does not have any significant positive or negative effect (44). In contrast, in cultured murine lymph node endothelium, which expresses high basal levels of VCAM-1 under static conditions, the application of relatively low levels of laminar shear stress (0–7 dynes/cm2) results in force- and time-dependent decreases in VCAM-1 mRNA (48). In the case of cultured murine endothelium, after cessation of flow VCAM-1 returns to its previous high baseline level. The existence of such complex temporal patterns of up- and/or down-regulation of steady-state message in response to a

relatively simple biomechanical stimulus, a continuous laminar shear stress of physiological amplitude, suggests that multiple, potentially distinct molecular mechanisms are involved in the regulation of these different endothelial genes. These patterns conceivably could reflect a complex interplay of shear-induced transcriptional activators and inhibitors as well as differential effects on transcript stability.

CHARACTERIZATION OF A POSITIVE SHEAR STRESS RESPONSE ELEMENT IN THE PDGF-B GENE

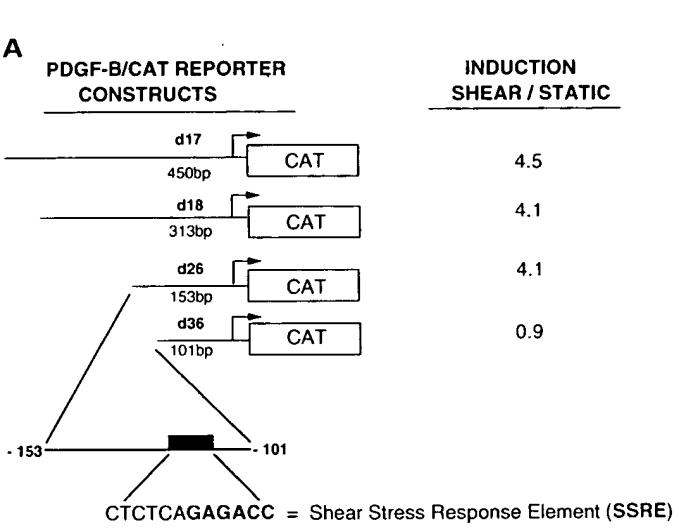
To more directly address the question of transcriptional regulation of endothelial gene expression by biomechancial forces, we have used a relatively simplified in vitro flow model, confluent bovine aortic endothelial monolayers (BAEC) exposed to a continuous laminar shear stress of physiological amplitude, 10 dynes/cm^2, in a cone-plate system and have analyzed PDGF-B gene expression, which in our hands exhibits an early positive pattern of induction (49). Northern blots showed increases in steady-state mRNA levels as early as 1 h after the onset of shear, and nuclear run-on assays documented the induction of transcription as early as 1 h. A reporter gene construct consisting of chloramphenicol acyltransferase (CAT) coupled to a 1.3 kb fragment of the human PDGF-B chain promoter was found to be responsive to laminar shear stress when transfected into BAEC. As illustrated in **Fig. 3*A***, further analysis using nested 5′ deletions of this promoter construct (d17, d18, d26, d36) identified a 52 bp region situated between position -153 and -101 (with respect to the transcriptional start site) that was necessary for shear stress responsiveness. Oligonucleotide probes spanning this region were then used in electromobility shift assays (EMSA) of nuclear extracts prepared from large samples (10^7 cells) of both static and shear-stressed endothelial monolayers. A strongly shear stress–inducible complex consistently formed in association with a 12 bp stretch within this region. Further mutational analyses defined a 6 bp core binding sequence, GAGACC, which was termed the shear stress response element or SSRE. Positive complex formation was observable with radiolabeled probes containing the SSRE as early as 30 min after the onset of flow (Fig. 3B). This rapid occurrence of nuclear protein-DNA binding was consistent with the kinetics of transcriptional activation of the intact PDGF-B gene, as well as the PDGF-B/CAT reporter construct.

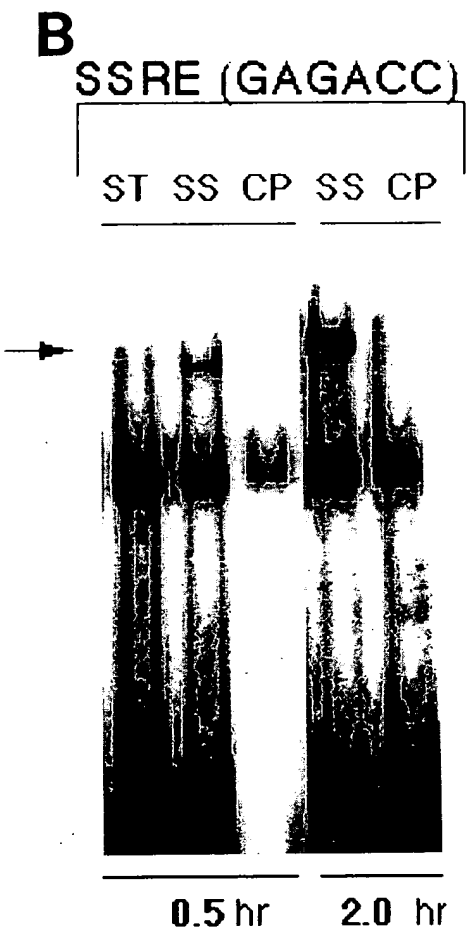
To test whether this SSRE, defined in the context of the intact human PDGF-B promoter, was potentially able to mediate the induction of other genes by shear stress, hybrid promoters consisting of the SSRE (GAGACC) or its complementary sequence (GGTCTC), coupled to a SV40-based enhancer-less promoter controlling a CAT reporter gene, were transfected into endothelial monolayers (Fig. 3*C*). Exposure to laminar shear stress resulted in activation of these SSRE-dependent hybrid promoters, thus establishing that this positive element was sufficient to confer shear responsiveness (50).

Because endothelial gene expression has also been shown to be responsive to another type of hemodynamic force, cyclic strain (26), we asked whether the SSRE might also play a role in this process. To test this hypothesis, endothelial cells were exposed to cyclic strain in a Flexercell system (10% biaxial strain, 60 cycles/min, 0.5–24 h), and their nuclei as well as nuclei from cells grown under static conditions were tested by EMSA. A highly inducible complex was formed between the SSRE probe and nuclear proteins from endothelial cells exposed to cyclic strain for as little as 30 min. This complex was specific and similar to the one formed with nuclear proteins from shear-stressed cells. Moreover, nuclear proteins derived from smooth muscle cells exposed to cyclic strain did not form this DNA-protein complex, thus suggesting a cell-type selectivity in the pathways leading to the activation of SSRE-containing genes by cyclic strain (50).

Computerized analysis of sequence databases revealed that several endothelial genes unrelated to PDGF that have been reported to be responsive to laminar shear stress also encode the SSRE (GAGACC) or its complementary sequence (GGTCTC) in their promoter regions (**Table 2**). Within a given gene (e.g., PDGF-B or tPA), this sequence appeared to be conserved among species. These findings thus further supported the hypothesis that the SSRE was part of a more general mechanism involved in the regulation of endothelial gene expression by biomechanical stimuli.

At the time of our initial analysis, ICAM-1, a member of the immunoglobulin superfamily of adhesion molecules was found to contain the SSRE within its core promoter region, whereas E-selectin and VCAM-1, two other endothelial-expressed adhesion molecules, did not. Although these three adhesion molecules had been shown to be coordinately induced in cultured endothelium by humoral stimuli such as cytokines and bacterial products, no comparative study of their regulation by hemodynamic forces had been reported. Exposure of cultured human umbilical vein endothelial cells to a physiologically relevant range of laminar shear stresses (2.5–46 dynes/cm^2) in the cone and plate apparatus illustrated in Fig. 1*B*, for intervals ranging from 4 to 48 h (45), resulted in significantly increased surface expression of ICAM-1 protein. This was correlated with increased adhesion of a JY lymphocytic cells, a human leukocyte line that expresses LFA-1, a counter-receptor for ICAM-1. Up–regulation of ICAM-1 transcript was detectable as early as 2 h after the onset of shear stress by Northern blot analysis. In contrast, E-selectin and VCAM-1 transcript and cell–surface protein were not up–regulated by shear stress at any time point examined, although they were strongly inducible by a cytokine stimulus (e.g., interleukin-1). This selective pattern of regulation of these endothelial adhesion molecules observed with shear stress thus correlates with the presence or absence of the SSRE in their promoters. Direct testing of the function of this element as a positive shear response regulator of the human ICAM-1 gene, using appropriate deletional reporter constructs, is currently in progress. It is interesting that when wall shear stresses in the rabbit carotid artery were increased or de-





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HYBRID PROMOTER CONSTRUCTS	INSERTED SEQUENCE	INDUCTION SHEAR / STATIC
SV40/CAT		0.9
SSRE-SV40/CAT	TCGGCTCTCAGAGACCCCCT	3.6
SSREc-SV40/CAT	TCGGCTCTCAGGTCTCCCCT	3.8
nonSSRE-SV40/CAT	TCGGCTCTCACTCTCACCCT	1.3

Figure 3. Characterization of a positive shear stress response element (SSRE) in the human PDGF-B gene. *A)* Deletional analysis of the human PDGF-B promoter, using CAT reporter genes transfected into static and shear-stressed endothelial monolayers, identifies a "shear stress response element" located approximately 100 basepairs upstream to the transcriptional start site (49). *B)* Electromobility shift assays (EMSA) reveal DNA binding proteins in shear-stressed endothelial nuclei that interact specifically with a radiolabeled probe encoding the SSRE (GAGACC). Positive SSRE-nuclear complexes (arrow) are detectable using nuclear extracts prepared as early as 30 min after the onset of the shear stimulus. (ST, static; SS, shear stress, 10 dynes/cm^2; CP, competition with unlabeled probe). *C)* Hybrid promoters containing the core-binding sequence of the SSRE (GAGACC) or its complementary sequence (GGTCTC) are sufficient to confer shear stress inducibility to a reporter gene transfected into endothelial cells. (SV40/CAT, enhancerless reporter construct; non–SSRE, irrelevant sequence).

creased for 5 days by surgical manipulations, a corresponding up- and down-regulation of ICAM-1 expression was observed in the endothelium using en face immunofluorescent staining (51).

TRANSCRIPTION FACTORS AND SHEAR STRESS

The identification of a *cis*-acting positive regulatory element in the PDGF-B gene promoter raised the more general question of the nature of the DNA binding proteins involved in transcriptional activation of this and other shear-inducible genes. Recent work in several laboratories has demonstrated that the intracellular concentration and activation state of a number of known transcription factors can be influenced by shear stresses in endothelial cells (3, 31). For example, the IERGs c-*fos*, c-*jun*, and c-*myc*, whose products can function as transactivating factors, are all induced in cultured endothelium by shear stress, although with different kinetics (3, 40, 41). Translocation of c-fos protein to the nucleus has been observed in endothelial cells exposed to "arterial levels" (25 dynes/cm^2) of laminar shear stress. Both the induction of transcription of the c-*fos* gene and the translocation of its protein product appear to involve protein kinase C, G–proteins, phospholipase C, and intracellular calcium (40). Nuclear extracts from shear-stressed endothelial cells form complexes with an AP-1 probe (which can interact with c-fos and/or c-jun proteins) in EMSA assays, with two discrete peaks of activity (20 min, 2 h) (52). A second transcription factor system, NFκB, which appears to play a central role in the regulation of multiple pathophysiologically relevent genes in vascular cells (see ref 53), is also activated by shear stress, as originally shown by Davies and co–workers (52) using EMSA analysis. Using immunofluorescence microscopy (**Fig. 4**), we have recently observed translocation of the heterodimeric components of the NFκB activation complex, p50/p65, from the cytoplasm to the nucleus as early as 10 min after exposure of both

TABLE 2. *Shear-stress-responsive endothelial genes containing the SSRE (GAGACC) or its complementary sequence (GGTCTC)*

Gene	Species	Location	Sequence*
PDGF-B	human	−125	GAGACC
	feline	−125	GAGACC
	murine	−125	GAGACC
tPA	human	−345	GAGACC
	murine	−252	GAGACC
	feline	−252	GAGACC
TGF−β1	human	−1219	GGTCTC
	murine	−401	GGTCTC
		−1314	GAGACC
MCP-1	human	−202	GGTCTC
		−1919	GAGACC
Endothelin-1	human	−236	GGTCTC
ec-NOS	human	−999	GGTCTC
		−1986	GAGACC
c-fos	human	−216	GAGACC
ICAM-1	human	−644	GAGACC

*Primary sequence data as cited in reference 49 and Genebank.

human and bovine endothelial cells to a laminar shear stress stimulus (10 dynes/cm^2). Although the core binding sequence defined by mutational analysis of the SSRE in the PDGF-B promoter did not correspond directly to any previously defined transcription factor binding site, it did bear some similarity to consensus sites for members of the NFκB/rel family (cf., ref 53). This led to the following studies of the role of NFκB in the transactivation of the SSRE, which have been undertaken in collaboration with the Collins laboratory (54). First, nuclear extracts from endothelial cells subjected to laminar shear stress were found to form complexes with an SSRE probe that were comparable on EMSA analysis to those formed with the functional NFκB binding site present in the E-selectin promoter. These shear-induced complexes contained both p65 and p50 subunits, as demonstrated by supershift with NFκB-specific antibodies. DNase I footprinting, with recombinant p50 and p65 proteins, further documented the interaction of these NFκB components with the SSRE region of the PDGF-B promoter. Mutation of the SSRE within the PDGF-B promoter abolished shear stress responsiveness of reporter gene constructs, as well as the ability to bind recombinant p50 and p65 proteins in EMSA assay. Cotransfection experiments with p50 and p65 expression constructs documented their ability to transactivate SSRE-dependent reporter genes. All of these findings are consistent with a functional role for the NFκB system in regulating SSRE-dependent gene expression. Further studies are necessary to define whether the binding of NFκB components to the SSRE is sufficient to activate the expression of an endogenous gene in response to a shear stimulus or if the participation of additional transcription factors is required.

COMPLEXITY OF TRANSCRIPTIONAL REGULATION BY SHEAR STRESS

The characterization of a *cis*–acting, shear-inducible positive regulatory element in the human PDGF-B gene and the implication of NFκB components in its transactivation provide a useful experimental model for further investigation. At the same time, it raises several interesting issues that illustrate the complexity of transcriptional regulation of endothelial genes by shear stress. For example, the adhesion molecules VCAM-1 and E-selectin, although lacking the SSRE, do contain multiple NFκB sites that appear to be functional in cytokine-induced transcription. Given that a shear stimulus is sufficient for NFκB activation and translocation to the endothelial nucleus, what is responsible for the lack of induction of these genes by shear? Second, the presence of the SSRE in a given gene does not necessarily indicate its functional importance in shear-induced transcriptional activation. Recently this has been experimentally demonstrated in the case of the MCP-1 gene (45), whose shear inducibility appears to be dependent on a TRE (AP-1 family) site rather than the two SSREs (cf., Table 2) present in its promoter. In addition, there are examples of shear-inducible endothelial genes such as PDGF-A (55) whose core promoter region, although responsive to shear stress, does not appear to contain the SSRE, thus implying the existence of other positive shear-responsive regulatory elements. Finally, shear stress also appears to be downregulating the expression of certain endothelial genes (ET-1, VCAM-1, cf. Fig. 2) at a transcriptional level. For example, recent studies by Malek and co–workers (38) have mapped a negative shear-responsive element in the preproendothelin-1 gene to a 400 bp region approximately 2 kb upstream from the transcriptional initiation site. Thus, the complex temporal patterns of endothelial gene regulation schematically represented in Fig. 2 may reflect a combinatorial complexity involving the synergism and/or antagonism of both positive and negative shear stress-responsive elements and their transactivating factors.

COMPLEXITY OF HEMODYNAMIC STIMULI IN VIVO

Throughout this review, we have limited our discussion of endothelial gene regulation by hemodynamic forces to a single type of stimulus, continuous laminar shear stress, applied to cultured endothelial monolayers. Although these simple in vitro model systems have yielded much valuable new information, they fail to mimic the complexities of the in vivo biomechanical milieu of endothelium in several important respects. First, virtually all in vitro flow studies involve an abrupt transition from an essentially no–flow (static) condition. Analogous situations are rarely encountered in vivo, possible exceptions being the neonatal pulmonary circulation after closure of the ductus arteriosis or certain vascular surgical manipulations (e.g., coronary artery bypass grafting). Second, in vitro studies have demonstrated flow accomodation as well as responsiveness to both

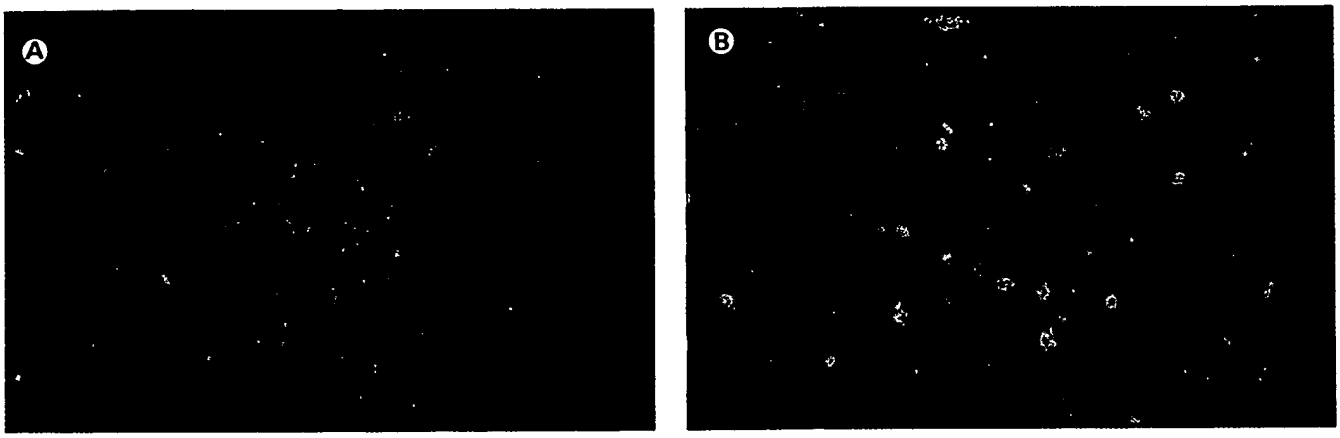

Figure 4. Nuclear translocation of NFκB by laminar shear stress. Immunofluorescence microscopy of NFκB component (p65) in confluent human endothelial monolayers. A) Static (no flow) monolayer. Note uniform cytoplasmic staining pattern and absence of nuclear staining. B) Shear-stressed (10 dynes/cm2) monolayer 10 min after onset of flow. Note positive nuclear staining, indicative of translocation of p65 from cytoplasm into nuclear compartment.

the onset and cessation of a flow stimulus (16), thus raising the question of the importance of flow preconditioning for in vitro experimental designs. Third, the in vivo pattern of atherosclerotic lesion localization strongly suggests an association of endothelial dysfunction with temporal and spatial complexities of blood flow. The in vitro generation of disturbed flow fields over endothelial monolayers (24) thus may provide a useful model with special pathophysiological relevance. Finally, although wall shear stress in particular has received much experimental attention, interactions with other hemodynamic parameters such as pressure and stretch remain largely unexplored. These reservations notwithstanding, it is likely that in vitro model systems will continue to provide useful insights into the molecular mechanisms linking biomechanical forces and gene expression in the endothelium.

SUMMARY AND FUTURE DIRECTIONS

The application of simple in vitro models has yielded new insights into how fluid mechanical forces act to regulate gene expression in vascular endothelium. In particular, the complex patterns of biological responses elicited by laminar shear stress are now being understood in terms of the activation of shear stress-responsive elements in the core promoters of various genes. These positive and negative transcriptional regulatory elements, through their combinatorial interactions with shear-induced transcription factors, thus provide a mechanism for coordinated patterns of response of otherwise unrelated genes in different physiological and pathophysiological settings.

But the basic question of how a frictional force, applied to the external surface of the luminal membrane of an endothelial cell, is translated into genetic regulatory events in its nuclear compartment still remains to be answered. Does this biomechanical transduction phenomenon use well–studied, receptor-activated second messenger cascades, or are novel pathways of intracellular signaling, conceivably with force transmission via cytoskeletal elements, involved (3, 31)? Do different biophysical stimuli elicit their biological responses via the same intracellular pathways and, ultimately, the same genetic regulatory mechanisms? The latter consideration clearly extends beyond endothelial cells and hemodynamic forces to the basic issue of how cells in general transduce biomechanical stimuli in their environment.

It is hoped that as the complexities of endothelial gene regulation by biomechanical forces are unraveled, we will arrive at a better working concept of the vascular endothelial cell as a dynamic integrator of local pathophysiologic stimuli within the vascular wall. Ultimately, this knowledge should contribute to our understanding of the pathogenesis of vascular disease and point to new therapeutic strategies. FJ

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